PCT 424 Rec'd PCT/PTO 24 JUL 2000

FORM PTO-1390 (REV 11-98)	U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 47-139
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO (If known, see 37 C.F.R. 1 5) 09/600911 Unassigned
INTERNATIONAL APPLICATION NO. PCT/AU99/00050	INTERNATIONAL FILING DATE 25 January 1999	PRIORITY DATE CLAIMED 23 January 1998 and 13 February 1998

TITLE OF INVENTION

PURIFICATION OF FIBRINOGEN

APPLICANT(S) FOR DO/EO/US

KANELLOS et al

OIPE JUL 24 2000 PATENT & TRADEMARK OFFICE

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ has been transmitted by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   - d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information. **References for IDS**

445052

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U.S. APPLICATION NO (If known, see 37 C.F.R 1 5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
Unassigned 09/600911	PCT/AU99/00050	47-139

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):	
-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $970.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $840.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2) paid to USPTO $690.00	
-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $670.00	
-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $96.00	

ENTER APPROPRIATE BASIC FEE AMOUNT =						$	970.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).						$	130.00	
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE				
Total Claims	14	-20 =	0	X	$18.00	$	0.00	
Independent Claims	1	-3 =	0	X	$78.00		0.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					$260.00	$	0.00	
TOTAL OF ABOVE CALCULATIONS =						$	**1100.00**	
Reduction by ½ for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 C.F.R. 1.9, 1.27, 1.28).							0.00	
SUBTOTAL =						$	**1100.00**	
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +							0.00	
TOTAL NATIONAL FEE =						$	**1100.00**	
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +						$	0.00	
Fee for Petition to Revive Unintentionally Abandoned Application ($1210.00 – Small Entity = $605.00)						$	0.00	
TOTAL FEES ENCLOSED =						$	**1100.00**	
						Amount to be: refunded	$	
						Charged	$	

a. ☒ A check in the amount of $1100.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
d. ☐ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201
Telephone: (703) 816-4000

SIGNATURE

Leonard C. Mitchard
NAME

29,009 July 24, 2000
REGISTRATION NUMBER Date

445052

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

KANELLOS et al

Atty. Ref.: 47-139

Serial No. Unassigned

Group:

Filed: July 24, 2000

Examiner:

For: PURIFICATION OF FIBRINOGEN

* * * * * * * * * * *

July 24, 2000

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above application as follows:

IN THE CLAIMS

Claim 3, line 1, delete "or claim 2".

Claim 6, line 1, delete "any one of claims 1 to 5" and replace by --claim 1--.

Claim 7, line 1, delete "any one of claims 1 to 6" and replace by --claim 1--.

Claim 8, line 1, delete "any one of claims 1 to 7" and replace by --claim 1--.

Claim 9, line 1, delete "any one of claims 1 to 8" and replace by --claim 1--.

Claim 10, line 1, delete "any one of claims 1 to 9" and replace by --claim 1--.

Claim 11, line 1, delete "any one of claims 1 to 10" and replace by --claim 1--.

Claim 13, line 1, delete "any one of claims 1 to 12" and replace by --claim 1--.

REMARKS

The above amendments have been made to place the application in a more traditional format.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____
Leonard C. Mitchard
Reg. No. 29,009

LCM:lks
1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

09/600911
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Purification of Fibrinogen

FIELD OF THE INVENTION

The present invention relates primarily to a method of obtaining fibrinogen. The method of the present invention also enables the obtaining of fibronectin and Factor XIII.

BACKGROUND OF THE INVENTION

The isolation of human fibrinogen has traditionally been carried out by classical plasma fractionation methods. Fibrinogen is precipitated from plasma either with ethanol (Blomback and Blomback, 1956), ammonium sulphate (Takeda, 1996), β alanine/glycine (Jakobsen and Kieruif, 1976), polymers (polyethelene glycol) and low ionic strength solutions (Holm, 1985) with relative high yield and homogeneity.

Further purification of fibrinogen precipitates can be achieved by ion-exchange chromatography conditions (Stathakis, 1978) and affinity chromatography (Kuyas, 1990). Specific contaminants can be absorbed out for example fibronectin an immobilised gelatine and plasminogen an immobilised lysine (Vuento, 1979).

Over the past few decades the overall structure and function of the fibrinogen molecule has been elucidated. The completion of the amino acid sequence of human fibrinogen (Henschen and Lottspeich, 1977) and the assignment of disulphide bonds (Blomback *et al.* 1976, Douma *et al.* 1978) provided data that confirmed the pioneer observations of an extended multidomained molecule (Hah and Slayter, 1959). The cloning of the fibrinogen genes and the complete amino acid sequence of all three chains of human fibrinogen from the cDNA studies are in agreement with those reported earlier based on the conventional amino acid sequencing procedures (Chung *et al.* 1983).

Precipitation methods are widely used for the manufacture of commercial fibrinogen. Chromatographic methods are now being explored as an alternative or to improve the purity of fibrinogen concentrates.

Fibrinogen interacts with a number of physiologically important proteins (Doolittle, 1984) such a plasminogen, thrombin, fibronectin, certain

strains of staphylococcal bacteria and platelets. A number of functional characteristics have been assigned to specific parts of the molecule including: the position of the fibrinopeptides released from the parent molecule by catalytic action of thrombin, fibrin covalent stabilisation donor and acceptor sites, carbohydrate clusters, polymerisation sites, calcium binding sites and the attachment sites for fibronectin, plasminogen, bacteria and platelets.

Human fibrinogen has a strong affinity for fibrin and this association has been exploited to affinity purify fibrinogen. Fibrin immobilised on Sepharose was used to isolate fibrinogen from human plasma (Matthias *et al.* 1975). Protein structure function studies have identified the peptide sequences in fibrin that have been shown to specifically bind to fibrinogen. Short peptides beginning with the sequence Gly-L-Pro-Arg have been shown to bind fibrinogen (Laudano and Doolittle, 1978). This sequence corresponds to the first three amino acids of the fibrin α-chain exposed by the thrombin catalysed release of the fibrinopeptide A in all vertebrate species. The addition of a second proline to this sequence was later shown to increase the affinity of the peptide Gly-Pro-Arg-Pro for fibrinogen almost ten-fold (Laudano and Doolittle, 1980). Based on this information, synthetic peptides corresponding to these sequences have also been shown to bind to fibrinogen (Gartner and Taylor, 1991).

SUMMARY OF THE INVENTION

The present invention relates to the large scale separation by precipitation of fibrinogen from other blood proteins in human blood plasma, cryoprecipitate, fraction 1 precipitate, other plasma fractions containing fibrinogen or fibrinogen containing culture media produced by recombinant DNA techniques and subsequent treatment of the heparin precipitate. The resultant fibrinogen-enriched preparation may be further purified to homogeneity utilising other precipitation methods, chromatographic steps such as ion-exchange chromatography, affinity chromatography size exclusion chromatography or ultrafiltration.

The present inventors have found that fibrinogen may be recovered from heparin precipitated paste, a by-product from the manufacturing process of Factor VIII (Antihaemophilic Factor, AHF). The heparin

precipitate paste may be solubilised with salt containing solutions such as NaCl to provide a fibrinogen preparation of high specific activity. The method of this invention has been shown to be superior to other known isolation procedures in that fibrinogen may be obtained with relative high yield and homogeneity from a discard fraction of processed plasma.

Accordingly, the present invention consists in a method of obtaining a fibrinogen enriched preparation, the method including the following steps:-

(i) adding an effective amount of a sulphated polysaccharide (SPS) to a fibrinogen containing solution with to form a fibrinogen containing precipitate; and

(ii) extracting fibrinogen from the fibrinogen containing precipitate from step (i) with a solution containing at least 0.1 M, and preferably at least 0.2M, salt to obtain a fibrinogen enriched preparation.

In a preferred embodiment of the present invention the solution includes at least one salt selected from the group consisting of chloride, phosphate and acetate salts, and more preferably includes NaCl. It is preferred that the NaCl is present at concentration of from about 0.1M to about 2.0M, preferably from about 0.2M to about 0.8M.

In a further preferred embodiment the solution includes ε-aminocaproic acid.

In another preferred embodiment the SPS is a heparinoid selected from the group consisting of mucopolysaccharide polysulphate, pentosan polysulphate, chondroitin sulphate, dextran sulphate and heparin and is preferably heparin.

The amount of SPS used can be readily determined, however, it is preferred that the SPS is added to the fibrinogen containing solution to provide a concentration of SPS of at least 0.15 mg/ml.

Where the fibrinogen is to be used therapeutically the fibrinogen will be subjected to a viral inactivation step(s). Such inactivation procedures are well known in the art and include heating and solvent detergent treatment.

The fibrinogen containing solution may be any of a number of such solutions well known to those skilled in the art such as plasma (including anti-coagulated plasma), plasma fractions (such as cryoprecipitate and solubilised fraction I) and fibrinogen-containing cell culture media arising from the production of fibrinogen by recombinant DNA techniques. It is,

however, preferred that the fibrinogen containing solution is a blood plasma fraction, preferably cryoprecipitate.

The fibrinogen may be further purified from the fibrinogen enriched preparation using any of a range of techniques well known to those skilled in this area. For example, purifying the fibrinogen from the fibrinogen enriched preparation by either reprecipitating the fibrinogen with a protein precipitant in the presence of salts and/or amino acids or by chromatographic techniques such as ion exchange, affinity, hydrophobic or gel permeation chromatography or a combination of both techniques. For use the fibrinogen enriched preparation will typically be treated to remove SPS and/or plasminogen. This can be achieved using a number of methods well known in the art. Examples of known purification methods include those described in the following references, the disclosures of which are incorporated herein by reference:-

"Affinity purification of human fibronectin on immobilized gelatine" Regnault V, Rivat C, & Stoltz; Journal of Chromatography, 432 (1988) 93-102

"Isolation of Fibronectin under Mild Conditions" Morgenthaler J, Baillod P & Friedli H; Vox Sang 47 (1984) 41-46

"Plasminogen: Purification from human plasma by affinity chromatography" Deutsch D & Mertz E; Science 170 (1970) 1095-1096

"A Pasteurised Concentrate of Human Plasma FactorXIII for Therapeutic Use" Winkelman L, Sims G, Haddon M, Evans D & Smith J; Thrombosis and Haemostasis 55(3)(1986) 402-405

"The Preparation of Human Fibrinogen Free of Plasminogen" Mosesson M; Biochim Biophys Acta 57 (1962) 204-213

US Patent No. 3,340,156

"Severely Heated Therapeutic Factor VIII Concentrate of High Specific Activity" Winkelman L, Owen n, Evans D, Evans H, Haddon M, Smith J, Prince P & Williams J; Vox Sang 57 (1989) 97-103

"Plasma Protein Fractionation" Heide K, Haupt H & Schwick H; in The Plasma Proteins, 2nd Edition Vol 3 (1977) Putnam F. (Ed)

Depending on the nature of the fibrinogen containing solution the fibrinogen enriched preparation may also contain fibronectin and Factor XIII.

For example, if the fibrinogen containing solution is plasma or a plasma fraction the fibrinogen enriched preparation will also contain fibronectin and/or Factor XIII. If desired these proteins may also be further purified from the fibrinogen enriched preparation using known separation techniques.

Accordingly the present invention also provides a method of obtaining a preparation enriched for fibronectin or Factor XIII, the method comprising extracting fibronectin or Factor XIII from the fibrinogen enriched preparation obtained according to the method of the present invention in which the fibrinogen containing solution is a blood plasma fraction.

As will be recognised from the above description the present invention provides a method of purifying fibrinogen from blood plasma concentrates especially cryoprecipitate. The most commercially important of the plasma concentrates currently used are the blood plasma fraction commonly known as cryoprecipitate and purified concentrates prepared from cryoprecipitate. Conventionally, cryoprecipitate is defined as a precipitate rich in Factor VIII and fibrinogen and which is prepared from frozen freshly prepared human plasma by a low temperature plasma fractionation technique.

Typically deep frozen plasma is softened at temperatures below 5°C and the Factor VIII rich cryoprecipitate is collected by centrifugation.

Cryoprecipitate prepared in this way has been used as a commercial source of Factor VIII and typically contained concentrated within it from 40 to 60% of the total amount of Factor VIII contained in the whole blood from which the plasma is derived. There have been numerous studies designed to improve the yield of Factor VIII from cryoprecipitate and to further purify it. The presence of high concentrations of fibrinogen and fibronectin in Factor VIII preparations is undesirable because they have been found to give rise to unacceptable losses of Factor VIII during some of the processing steps. Fibrinogen is of particular concern because it is normally present in much greater concentrations than fibronectin in blood plasma and cryoprecipitate and is usually more difficult to remove than fibronectin.

A method of preparing a Factor VIII containing preparation which includes the steps of precipitating fibrinogen and fibronectin from a buffered solution of a Factor VIII containing blood plasma fraction by the addition of a sulphated polysaccharide is disclosed by Winkleman (AU B55435/86) who

described one method of purifying Factor VIII in which the fibrinogen is precipitated from a buffered solution of cryoprecipitate held at pH 6 to 8.

The heparin precipitate is removed from the Factor VIII containing supernatant and discarded. The present invention enables the purification of valuable proteins from this previous waste product.

DETAILED DESCRIPTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following examples.

MATERIALS

BUFFERS

Tris buffer:

50 mM Tris
5 mM EDTA
5 mM ε-aminocaproic acid
0.8 M NaCl
pH 7.3

Citrate buffer:

20 mM Na-Citrate
5 mM EDTA
5 mM ε-aminocaproic acid
0.8 M NaCl
pH 7.3

Tris wash buffer:

50 mM Tris
5 mM EDTA
5 mM ε-aminocaproic acid
pH 7.3

Heparin paste is a by-product from the manufacture of AHF (High Purity). It is obtained from production and then stored in 100g aliquots at -80°C.

METHOD

Buffer Optimisation Study

Heparin paste was thawed in a 37°C water bath and allowed to equilibrate to room temperature for 30 minutes. Heparin paste (6 g) was added to 50 ml of the extraction buffer. This was incubated at room temperature for 30 minutes, stirring at a rate where no frothing occurs. The solubilised paste was centrifuged at 4,200 rpm for 10 minutes at 4°C. The mass of both the pellet and supernatant was determined and the pellet was discarded. The supernatant was aliquoted (5 ml) and then frozen at -80°C.

The initial extraction of fibrinogen from Heparin paste was performed using Tris buffer (50 mM Tris, 5 mM EDTA, 5 mM ε-aminocaproic acid, 0.8 M NaCl, pH 7.3)(CSL work book 0525 pp 28-47). In these experiments, buffer components and conditions were altered to optimise the extraction procedure. Variations on the Tris buffer included:

- removing one component of the Tris buffer and keeping all others constant
- varying the pH of the Tris buffer, and
- varying the NaCl concentration of the Tris buffer.

Variations of buffer components and conditions of Citrate buffer (20 mM Na-Citrate, 5 mM EDTA, 5 mM ε-aminocaproic acid, 0.8 M NaCl, pH 7.3) were also examined for their ability to extract fibrinogen from Heparin paste.

Variations on the Citrate buffer included:

- removing one component of the Citrate buffer and keeping all others constant
- varying the pH of the Citrate buffer
- varying the NaCl concentration of the Citrate buffer minus EDTA, and
- varying the Na-Citrate concentration of the Citrate buffer.

Water for Injection BP (WFI) and WFI with 0.8 M NaCl were also examined for their ability to extract fibrinogen from Heparin paste.

The ability of each buffer to extract fibrinogen from Heparin paste was determined by analysis of total clottable fibrinogen, total protein and factor XIII activity in the supernatant.

Heparin Paste Concentration Study

The maximum amount of Heparin paste able to be solubilised in a constant volume of Tris buffer was determined. Heparin paste (obtained from production and processed immediately) was weighed out (6 g, 22 g and 40 g) and added to 50 ml of the Tris wash buffer. This was incubated at room temperature for 30 minutes, stirring at a rate where no frothing occurs. The paste was then separated from the wash buffer and transferred to a beaker containing 50 ml of Tris (extraction) buffer. This was incubated at room temperature for 30 minutes, stirring at a rate where no frothing occurs. The solubilised paste was centrifuged at 4,200 rpm for 10 minutes at 4°C. The mass of both the pellet and supernatant was determined and the pellet was discarded. The supernatant was aliquoted (5 ml) and then frozen at -80°C.

The ability of Tris buffer (constant volume) to extract fibrinogen from different amounts of Heparin paste was determined by analysis of total clottable fibrinogen, total protein, factor XIII activity and total plasminogen in the supernatant.

RESULTS

BUFFER OPTIMISATION STUDY

Experiment 1:

Experiment 1 showed that solubilisation of Heparin paste using Tris buffer resulted in the extraction of 14.1 mg/ml protein, with 75% being clottable fibrinogen (Table 1).

Heparin paste was also solubilised by Tris buffer minus specific components. For these buffers, extracted protein ranged from 14.2 mg/ml to 16.5 mg/ml, with 52-83% clottable fibrinogen being recovered (Table 1). The greatest amount of clottable fibrinogen, 83%, was extracted by solubilising Heparin paste in Tris buffer minus EDTA. Only 52% of clottable fibrinogen was extracted by solubilising Heparin paste in Tris buffer minus ε-aminocaproic acid and 66% when Tris buffer minus Tris was used (Table 1).

Table 1: Fibrinogen extraction from Heparin paste using Tris buffer minus specific components.

Buffer	Heparin Paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total)% clottable
Tris buffer	6.1	53.5	1.0	14.1 (754.4)	10.6 (567.1) 75%
Tris buffer minus Tris	6.1	54.2	1.1	14.2 (769.6)	9.3 (504.1) 66%
Tris buffer minus EDTA	6.3	53.3	1.3	16.5 (879.5)	13.7 (730.2) 83%
Tris buffer minus ε-amino - caproic acid	6.0	54.2	1.1	14.9 (807.6)	7.7 (417.3) 52%

Experiment 2:

Experiment 2 showed that solubilisation of Heparin paste by Tris buffer (pH 6.0 to pH 8.0) resulted in comparable extracted protein levels, 15.0 mg/ml (Tris buffer pH 7.5) to 17.3 mg/ml (Tris buffer, pH 7.0). The proportion of clottable fibrinogen ranged from 48% (Tris buffer pH 8.0) to 60% (Tris buffer pH 7.3) (Table 2).

Table 2: Fibrinogen extraction from Heparin paste using Tris buffer at various pH levels.

Sample	Heparin paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total) % clottable
pH 6.0	6.2	55.7	1.0	16.3 (907.9)	9.2 (512.4) 56%
pH 6.5	6.3	54.9	1.1	16.3 (894.9)	9.6 (527.0) 59%
pH 7.0	6.4	52.4	1.4	17.3 (906.5)	9.1 (476.8) 53%
pH 7.3	6.0	53.2	1.4	15.9 (845.9)	9.6 (510.7) 60%
pH 7.5	6.2	56.7	1.4	15.0 (850.5)	8.6 (487.6) 57%
pH 8.0	6.3	52.7	1.2	16.7 (880.1)	8.0 (421.6) 48%

Experiment 3:

Table 3 shows data obtained when Tris buffer, with varying concentrations of NaCl (0.2 M-2 M), were used for the extraction of fibrinogen from Heparin paste. The absence of NaCl resulted in partial solubilisation of the Heparin paste (5.3 g Heparin paste unsolubilised). Consequently, low levels of both protein and fibrinogen were recovered: 1.9 mg/ml of protein extracted with only 11% being clottable fibrinogen. The amount of protein extracted using Tris buffer with NaCl, 0.2 M-2 M, was comparable. The greatest level of clottable fibrinogen was obtained when

Tris buffer containing 0.6 M NaCl was used (8.4 mg/ml). The percentage of clottable fibrinogen decreased with increasing salt concentration (greater than 0.6 M NaCl).

Table 3: Fibrinogen extraction from Heparin paste using Tris buffer with various NaCl concentrations.

Sample NaCl Concentration	Heparin paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total) % clottable
0.0 M	6.0	49.1	5.3	1.9 (93.3)	0.2 (9.82) 11%
0.2 M	6.1	53.3	1.4	14.9 (794.2)	6.8 (362.4) 46%
0.4 M	6.1	55.1	1.1	15.8 (870.6)	7.8 (429.8) 49%
0.6 M	6.0	54.2	0.9	15.6 (845.5)	8.4 (455.3) 54%
0.8 M	12.1 (100 ml buffer)	104.9	1.7	16.9 (1772.8)	7.0 (734.3) 41%
1.0 M	6.1	54.8	1.5	14.1 (772.7)	5.9 (323.3) 44%
1.5 M	6.0	57.2	1.3	15.5 (886.6)	6.2 (354.6) 40%
2.0 M	6.0	55.1	1.7	15.7 (865.1)	4.5 (248.0) 29%

Experiment 4:

Experiment 4 examined the effect of Citrate buffer on the solubilisation of three batches of Heparin paste. The level of protein extracted from Heparin paste using Citrate buffer was 14.7 mg/ml, 14.0 mg/ml and 17.4 mg/ml (Tables 4a, 4b and 4c respectively). Of this protein, 91% (Table 4a), 65% (Table 4b) and 95% (Table 4c) was clottable fibrinogen. Analysis of Heparin paste solubilised using Citrate buffer for factor XIII showed 5.2 IU/ml, 9.5 IU/ml and 8.4 IU/ml (Tables 4a, 4b and 4c, respectively).

Heparin paste was also solubilised by Citrate buffer minus specific components. The removal of EDTA from Citrate buffer had no effect on extraction of protein, clottable fibrinogen and factor XIII from Heparin paste (14.6 mg/ml, 99% and 4.3 IU/ml respectively, Table 4a, 13.2 mg/ml, 113% and 6.3 IU/ml, respectively, Table 4b, 14.5 mg/ml, 106% and 4.2 IU/ml, respectively, Table 4c). The removal of ε-aminocaproic acid also showed no effect on protein, clottable fibrinogen and factor XIII from Heparin paste (14.6 mg/ml, 86% and 3.9 IU/ml respectively, Table 4a, 14.5 mg/ml, 65% and 8.0 IU/ml, respectively, Table 4b, 14.7 mg/ml, 72% and 7.8 IU/ml, respectively, Table 4c). However, Citrate buffer without NaCl was not able to solubilise the Heparin paste resulting in undetectable levels of protein and fibrinogen in the supernatant. The use of Na-Citrate alone was also unable to extract protein, factor XIII or fibrinogen, from the three Heparin paste batches (Tables 4a, 4b and 4c).

Table 4a: Fibrinogen extraction from Heparin paste using Citrate buffer minus specific components.

Sample	Heparin Paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total) % clottable	FXIII IU/ml (total)
Citrate buffer	6.2	53.3	2.2	14.7 (783.5)	13.4 (714.2) 91%	5.2 (277.2)
Citrate buffer minus EDTA	6.0	52.3	2.8	14.6 (763.6)	14.5 (758.4) 99%	4.3 (224.9)
Citrate buffer minus ε-amino -caproic acid	6.2	53.0	1.6	14.6 (773.8)	12.5 (662.5) 86%	3.9 (206.7)
Citrate buffer minus NaCl	6.2	48.1	5.1	undetect -able	undetectabl e	NA
20 mM Na-Citr ate	6.1	46.0	7.3	undetect -able	undetectabl e	NA

Table 4b: Fibrinogen extraction from Heparin paste using Citrate buffer minus specific components.

Sample	Heparin Paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total) % clottable	FXIII IU/ml (total)
Citrate buffer	6.1	53. 6	2.5	14.0 (750.4)	9.1 (487.8) 65%	9.5 (509.2)
Citrate buffer minus EDTA	6.0	53.9	2.5	13.2 (711.5)	14.9 (803.1) 113%	6.3 (339.6)
Citrate buffer minus ε-amino caproic acid	6.1	53.4	2.4	14.5 (774.3)	9.4 (502.0) 65%	8.0 (427.2)
Citrate buffer minus NaCl	6.1	48.1	7.2	undetectable	undetectable	NA
20 mM Na-Citrate	6.0	46.9	6.5	undetectable	undetectable	NA

Table 4c: Fibrinogen extraction from Heparin paste using Citrate buffer minus specific components.

Sample	Heparin Paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total) % clottable	FXIII IU/ml (total)
Citrate buffer	6.0	45.1	2.2	17.4 (784.7)	16.5 (744.2) 95%	8.4 (378.8)
Citrate buffer minus EDTA	6.1	53.2	1.9	14.5 (771.4)	15.4 (819.3) 106%	4.2 (223.4)
Citrate buffer minus ε-amino -caproic acid	6.1	52.7	1.8	14.7 (774.7)	10.5 (555.4) 72%	7.8 (411.1)
Citrate buffer minus NaCl	6.1	41.3	7.0	undetectable	undetectable	NA
20 mM Na-Citrate	6.1	45.3	6.9	undetectable	undetectable	NA

Experiment 5:

The pH optimisation for the Citrate buffer involved testing a pH range for 5.0 to 9.0. For pH ranges 6.0 to 9.0, protein and clottable fibrinogen levels extracted were comparable (13.5 mg/ml to 14.2 mg/ml and 85% to 94%). However, at pH 5.0, only 6.0 mg/ml of protein was extracted with 20% clottable fibrinogen (Table 5).

Table 5: Fibrinogen extraction from Heparin paste using Citrate buffer at various pH levels.

Sample	Heparin paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total protein)	Fibrinogen mg/ml (total) % clottable
pH 5.0	6.1	51.9	3.8	6.0 (311.4)	1.2 (62.3) 20%
pH 6.0	6.1	53.5	2.1	13.9 (743.7)	13.1 (700.9) 94%
pH 7.0	6.1	54.9	2.1	14.2 (779.6)	12.0 (658.8) 85%
pH 7.3	6.0	54.5	2.3	13.8 (752.1)	12.4 (675.8) 90%
pH 8.0	6.1	54.1	2.1	13.6 (735.8)	11.9 (643.8) 87%
pH 9.0	6.1	53.7	2.0	13.5 (725.0)	11.9 (639.0) 88%

Experiment 6:

Citrate buffer minus EDTA and containing a range of NaCl concentrations (0 M-1 M) was used in experiment 6. Two experiments were performed to test this range of concentrations. Results from this experiment showed that the solubility of Heparin paste increases with increasing NaCl concentration in the Citrate buffer minus EDTA (Table 6). Heparin paste was found to be only sparingly soluble in Citrate buffer minus EDTA with 0.05 M or less NaCl. This is seen by the extraction of only 0.2 mg/ml-0.4 mg/ml protein and 0.3 mg/ml-0.6mg/ml clottable fibrinogen. In two different experiments the presence of 0.1 M NaCl in the buffer showed a range of 4.2 mg/ml-11.0 mg/ml protein (86%-105% clottable fibrinogen) was extracted. This increased to 12.3 mg/ml-14.7 mg/ml protein (107%-119% clottable fibrinogen) when 0.2 M NaCl or greater is present. A constant level of extracted protein and clottable fibrinogen was demonstrated by addition of increasing concentrations of NaCl (0.4 M-1.0 M) to the Citrate buffer minus EDTA. These levels are comparable to the control, Citrate buffer minus EDTA containing 0.8 M NaCl. The levels of factor XIII ranged from undetected to approximately 3 IU/ml when 0 M-0.1 M NaCl was incorporated into the buffer. When NaCl (0.2 M-1 M) was added to the Citrate buffer minus EDTA, factor XIII levels remained constant. A maximum value of 7.8 IU/ml factor XIII was obtained when 0.4 M NaCl was added to the Citrate buffer minus EDTA.

Table 6: Fibrinogen extraction from Heparin paste using Citrate buffer minus EDTA with various NaCl concentrations.

Sample	Heparin Paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total)% clottable	FXIII IU/ml (total)
0.0 M	6.0	47.8	7.2	0.2 (9.6)	0.3 (14.34) 149%	NA
0.05 M	6.0	47.9	7.3	0.4 (19.2)	0.6 (28.7) 149%	NA
0.1 M NaCl minus EDTA	6.0	48.7	6.4	4.2 (204.5)	3.6 (175.3) 86%	3.0 (146.1)
	6.0	51.6	3.3	11.0 (567.6)	11.6 (598.6) 105%	3.3 (170.3)
0.15 M	6.0	52.8	2.3	12.8 (675.8)	14.3 (755.0) 115%	3.2 (169.0)
0.2 M NaCl minus EDTA	6.1	53.0	2.7	12.3 (651.9)	13.2 (699.6) 107%	7.2 (381.6)
	6.0	53.6	1.9	14.7 (787.9)	17.5 (938.0) 119%	3.1 (166.2)
0.4 M NaCl minus EDTA	6.0	52.8	3.1	13.8 (728.6)	15.0 (792.0) 109%	7.8 (411.8)

Sample	Heparin Paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total)% clottable	FXIII IU/ml (total)
0.6 M NaCl minus EDTA	6.1	55.0	2.0	14.3 (786.5)	14.5 (797.5) 101%	4.8 (264.0)
0.8 M NaCl (control) minus EDTA	6.1	54.7	2.2	14.1 (771.3)	14.1 (771.3)[b] 100%	7.3 (399.3)
1 M NaCl minus EDTA	6.1	55.3	2.2	14.0 (774.2)	12.5 (691.3) 89%	6.1 (337.3)

Experiment 7:

Table 7 details the levels of protein and clottable fibrinogen recovered by varying the levels of Na-Citrate in the Citrate buffer (Experiment 7). As the Na-Citrate concentration increased from 5 to 80 mM, there was a slight increase in the level of protein extracted (13.4 mg/ml minimum, 15.1 mg/ml maximum) (Table 7). The levels of clottable fibrinogen increased, from 79% to 107%, as the amount of Na-Citrate increased to 20 mM (Table 7). A decline in the amount of clottable fibrinogen was observed when 40 mM and 80 mM Na-Citrate was added to the buffer (85% and 74%, respectively) (Table 7).

Table 7: Fibrinogen extraction from Heparin paste using Citrate buffer with various levels of Na-Citrate.

Sample	Heparin paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total) % clottable
5.0 mM	5.6	54.3	1.9	13.4 (727.6)	10.6 (575.6) 79%
10.0 mM	6.1	52.7	2.2	14.7 (774.7)	13.1 (690.4) 89%
15.0 mM	6.3	54.9	1.9	14.8 (812.5)	12.4 (680.8) 84%
20.0 mM	6.2	53.3	2.2	14.7 (783.5)	15.7 (836.8) 107%
40.0 mM	6.1	53.4	2.0	15.0 (801.0)	12.7 (678.2) 85%
80.0 mM	6.1	55.1	1.5	15.1 (832.0)	11.2 (617.1) 74%

Experiment 8:

Fibrinogen extraction was also performed using WFI at pH 7.3. Although the amount of total protein extracted was low, 3.3 mg/ml, all was shown to be clottable fibrinogen (106%) (Table 8). Higher levels of protein (10.1 mg/ml) were recovered when 0.8 M NaCl (dissolved in WFI) was used as the extraction buffer (Table 8). Of this protein, 84% was clottable fibrinogen.

Table 8: Fibrinogen extraction from Heparin paste using WFI.

Sample	Heparin paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total) % clottable
WFI pH 7.3	6.1	56.2	1.6	3.3 (185.5)	3.3 (185.5) 100%
WFI containing 0.8 M NaCl	6.4	53.3	1.5	10.1 (538.3)	8.5 (453.1) 84%

Experiment 9

HEPARIN PASTE CONCENTRATION STUDY

The amount of Heparin paste that could be solubilised by a constant volume of Tris buffer pH 7.3 was determined. Total protein extracted from 6 g of Heparin paste with 50 ml Tris buffer ranged from 14.9 mg/ml to 17.9 mg/ml containing 55%-71% clottable fibrinogen. The amount of active factor XIII extracted from this amount of Heparin paste ranged between 4.5 IU/ml to 6.2 IU/ml. This amount of solubilised Heparin paste was found to contain 14.1 μg/ml to 16.6 μg/ml plasminogen (Table 9).

When the mass of Heparin paste was increased to 22 g, the amount of protein extracted was 38.6 mg/ml with 58% attributed to clottable fibrinogen. The amount of plasminogen and factor XIII extracted was 18.6 μg/ml and 26.0 IU/ml, respectively (Table 9).

When solubilising approximately 40 g of Heparin paste, the amount of protein extracted ranged between 48.0 mg/ml and 53.1 mg/ml (Table 9). Of this, 56% to 79% was clottable fibrinogen. The amount of active factor XIII extracted from this amount of Heparin paste ranged between 24.6 IU/ml to 27.5 IU/ml. This amount of solubilised Heparin paste was found to contain 21.5 μg/ml to 24.4 μg/ml plasminogen (Table 9).

Table 9: Fibrinogen extraction from 6 g, 22 g and 40 g Heparin paste using a constant volume of Tris buffer pH 7.3.

Sample	Heparin Paste (g)	Supernatant (ml)	Pellet (g)	Protein mg/ml (total)	Fibrinogen mg/ml (total) % clottable	FXIII IU/ml (total)	Plasminogen µg/ml (total)
HepAS01-A1a	6.0	55.1	1.1	14.9 (821.0)	10.6 (584.1) 71%	5.7 (314.1)	15.2 (837.5)
HepAS02-A1b	6.5	53.8	1.4	15.4 (828.5)	10.3 (554.1) 67%	5.1 (274.4)	14.1 (758.6)
HepAS02-A1a	6.0	55.3	1.4	17.3 (956.7)	9.6 (530.9) 55%	6.2 (342.9)	15.5 (857.2)
HepAS02-A1b	6.0	56.1	1.3	17.9 (1004.2)	10.4 (583.4) 58%	4.5 (252.5)	16.6 (931.3)
HepAS02 A1HSB2	22.0	58.2	14.2	38.6 (2246.5)	22.4 (1303.7) 58%	26.0 (1513.2)	18.6 (1082.5)
HepAS02 A1HSB1	41.0	84.6	6.3	53.1 (4492.3)	30.0 (2538.0) 56%	25.0 (2115.0)	21.5 (1818.9)
HepAS01 A1HSB	40.4	76.6	13.4	52.0 (3983.2)	40.8 (3125.3) 78%	27.5 (2106.5)	24.4 (1869.0)
HepAS01 A2HSB	40.3	70.2	17.4	48.0 (3369.6)	38.4 (2695.7) 79%	24.6 (1726.9)	24.3 (1705.9)

EXPERIMENT 10

To demonstrate the applicability of the present invention to commercial application experiments were conducted using large quantities of heparin paste.

Results

Table 10 shows the results of the characterisation of fibrinogen extracted from heparin paste derived from the AHF(HP) process. This process involves dissolving cryoprecipitate in Tris buffer containing 0.8mg/mL of heparin to form a heparin paste. Six batches of AHF(HP) heparin paste produced from plasma, ranging from 1733 kg to 2618 kg, were extracted using 20 mM Tri-sodium Citrate buffer containing 400mM NaCl and 5mM ε-aminocaproic acid. Total protein extracted ranged from 14.1 mg/mL - 16.7 mg/mL of which 79% - 85% was clottable protein. Levels of fibronectin and plasminogen averaged 3.9 mg/mL and 46.1 μg/mL, respectively.

Table 11 shows the results of the characterisation of fibrinogen extracted from heparin paste derived from the Biostate process using 20 mM Tri-sodium Citrate buffer containing 400mM NaCl and 5mM ε-aminocaproic acid. (The Biostate process involves dissolving cryoprecipitate in water including a low level of heparin and the adding further heparin to a concentration of about 1.0 mg/mL to obtain a heparin paste). The total mass of plasma used to produce this heparin paste ranged from 1556 kg - 1635 kg. Total protein extracted ranged from 11.5 mg/mL - 12.8 mg/mL of which 71% - 85% was clottable protein. Levels of fibronectin and plasminogen averaged 2.75 mg/mL and 34.4 μg/mL, respectively.

Table 12 details the yield of fibrinogen obtainable from each kilogram of plasma. Purification of fibrinogen from heparin paste derived from the AHF(HP) process yields an average of 0.68 g per kg of plasma (range 0.62 g - 0.79 g). Purification of fibrinogen from heparin paste derived from the Biostate process yields an average of 0.425 g per kg of plasma (range 0.4 g - 0.45 g).

Table 10. Characterisation of solubilised heparin paste from cryoprecipitate extracted with Tris buffer

Batch No.	Heparin paste mass (kg)	Vol. of SHP (L)	Total Protein mg/ml	Fibrinogen mg/mL	Clottable protein	Fibrinogen g/kg paste	Fibronectin mg/mL	Plasminogen µg/mL
2	11.9	111.4	16.1	12.7	79	118.3	4.1	56.5
3	9.8	91.0	16.1	13.1	82	121.7	4.3	56.9
4	15.6	145.6	14.8	12.1	82	112.2	3.4	48.0
5	15.6	145.4	16.7	14.2	85	132.4	3.9	43.0
8	17.2	159.9	14.1	11.2	79	103.9	4.2	34.1
9	10.9	101.4	14.6	12.1	83	112.7	3.6	38.1

Table 11. Characterisation of solubilised heparin paste from cryoprecipitate extracted with water

Batch No.	Heparin paste mass (kg)	Vol. of SHP (L)	Total Protein mg/ml	Fibrinogen mg/mL	Clottable protein	Fibrinogen g/kg paste	Fibronectin mg/mL	Plasminogen µg/mL
6	7.8	72.6	12.8	9.1	71	84.4	3.1	35.5
7	7.85	73.0	11.5	9.5	82	88.2	2.4	33.3

Table 12. Yield of fibrinogen from plasma

Batch No.	Mass of plasma (kg)	Fibrinogen in SHP	g fibrinogen/kg plasma
2	2277	1359	0.62
3	1733	1192	0.69
4	2491	1762	0.71
5	2605	2065	0.79
8	2618	1791	0.68
9	1949	1227	0.63
6	1635	661	0.4
7	1556	693	0.45

Fibrinogen has been extracted from heparin paste that was derived from cryoprecipitate extracted in Tris buffer or in water. This process has been performed at a large scale (1.5 - 2.6 tonne of plasma). This preparation contains very high levels of fibrinogen with up to 90 % clottable protein. In addition, the extracted material contains fibronectin, plasminogen and factor XIII.

As is demonstrated this method of extracting fibrinogen from heparin paste will be useful in the large scale manufacture of fibrinogen, fibronectin, plasminogen and factor XIII.

The first experiment aimed at determining which components of Tris buffer pH 7.3 (control buffer) were necessary for Heparin paste solubilisation and fibrinogen extraction. The removal of Tris, EDTA and ε-aminocaproic acid from the buffer did not affect Heparin paste solubilisation compared to the control. A difference however, was observed in the amount of clottable fibrinogen extracted by Tris buffer minus EDTA and Tris buffer minus ε-aminocaproic acid. Removal of EDTA from the buffer resulted in increased levels of extracted clottable fibrinogen compared to the control and other combinations of Tris buffer. This decrease in fibrinogen extracted by EDTA containing buffers may be due to assay inhibition caused by the EDTA.

Exclusion of ε-aminocaproic acid appeared to reduce clottable fibrinogen by 23% and may be an important component of the Heparin paste Tris extraction buffer.

Variation of the pH of the Tris buffer did not demonstrate any observable differences in Heparin solubilisation or fibrinogen extraction. The pH of the Tris buffer was thus maintained at pH 7.3.

The absence of salt such as NaCl from Tris buffer resulted in partial solubilisation of the Heparin paste. Consequently, low levels of clottable fibrinogen were recovered. The addition of 0.2 M NaCl to the buffer demonstrated an increase in clottable fibrinogen. Heparin paste solubilisation thus requires the presence of a salt such as NaCl for complete solubilisation and hence extraction of clottable fibrinogen. The amount of clottable fibrinogen was generally constant and comparable to the control (Tris buffer containing 0.8 M NaCl) for buffers containing 0.2 M-1.5 M NaCl. A sharp decline in clottable fibrinogen was observed for Tris buffer containing 2 M NaCl. Therefore, concentrations of NaCl greater than 1.5 M may affect the amount of clottable fibrinogen extracted from Heparin paste, or interfere with the assay.

Citrate buffer was also examined for its ability to solubilise and extract fibrinogen from Heparin paste compared to Tris buffer. Three different batches of Heparin paste were used for the Citrate buffer experiments. Citrate buffer pH 7.3 was able to extract greater than 90% clottable fibrinogen representing an increase in clottable fibrinogen, compared to Tris buffer (75%). Similar results to the Tris buffer component study were also seen when EDTA and ε-aminocaproic acid were removed from the Citrate extraction buffer. Again, increased clottable fibrinogen (approximately 100%) was observed when Citrate buffer minus EDTA was used to solubilise Heparin paste. This shows that EDTA is not necessary for fibrinogen extraction and that EDTA-containing buffers may interfere with the fibrinogen assay.

The use of Na-Citrate alone was ineffective in resolubilising the Heparin paste and therefore, protein and fibrinogen levels were undetectable. Variation of the Na-Citrate concentration (5 mM-80 mM) of the Citrate buffer showed 20 mM recovered the greatest amount of clottable fibrinogen. The amount of Na-Citrate in the Citrate buffer was thus maintained at this concentration.

Optimisation of Citrate buffer pH revealed that pH 6.0-9.0 did not affect the level of clottable fibrinogen extracted from Heparin paste. A difference was observed when pH 5.0 was used. The amount of protein extracted was less than half of that obtained from other pH levels tested and clottable fibrinogen fell to 21%, suggesting that fibrinogen activity was affected at this pH. At these pH levels, the Citrate buffer was able to extract approximately 90% clottable fibrinogen whereas Tris buffer extracted approximately 60%.

As observed in the Tris buffer studies, salt such as NaCl is also required in the Citrate buffer minus EDTA for solubilisation of Heparin paste and extraction of clottable fibrinogen. Complete removal of NaCl from the Citrate buffer minus EDTA resulted in decreased solubility of the Heparin paste. This decrease in solubility decreased protein and clottable fibrinogen extraction. Analysis of NaCl levels (0-0.2 M) showed very little protein was extracted from Heparin paste using Citrate buffer minus EDTA containing less than 0.1 M NaCl. Of this extracted protein, all was shown to be clottable fibrinogen. At least 0.15 M NaCl, in Citrate buffer minus EDTA pH 7.3, was required to extract greater levels of protein and thus fibrinogen from the Heparin paste. Increased NaCl, 0.1 M-0.2 M, resulted in increased protein and fibrinogen extraction, suggesting that at least 0.2 M NaCl is required for optimal solubilisation of Heparin paste by Citrate buffer minus EDTA. As the concentration of NaCl, within the Citrate buffer minus EDTA, increased from 0.15 M-0.6 M, so did the amount of protein extracted from the Heparin paste. When raised from 0.6 M to 1 M, no further increases in extracted protein were demonstrated. The amount of clottable fibrinogen extracted from the Heparin paste also increased with increasing NaCl concentrations (0.2 M-0.8 M). Levels of clottable fibrinogen extracted from Heparin paste decreased when concentrations of NaCl greater than 0.8 M were incorporated into the Citrate buffer minus EDTA. This suggests that the optimal concentration range of NaCl required for protein and clottable fibrinogen extraction is 0.2 M-0.8 M. This concentration of NaCl was also found to be necessary for the efficient solubilisation of Heparin paste by Tris buffer.

WFI, pH 7.3, with and without 0.8 M NaCl, was also examined for its ability to solubilise and extract fibrinogen from Heparin paste. This study demonstrated that Heparin paste was soluble in water and did not require a buffered solution for solubilisation. WFI, pH 7.3, extracted only low protein

levels, however, all of this protein was shown to be fibrinogen. Again, the addition of greater than 0.2 M NaCl was necessary for increased extraction of protein and fibrinogen. WFI containing 0.8 M NaCl extracted comparable levels of fibrinogen compared to Tris buffer, pH 7.3, but lower levels compared to Citrate buffer.

The Heparin paste concentration study demonstrated that 6 g, 22 g, and 40g Heparin paste were able to be solubilised by 50 mL Tris extraction buffer. Increasing levels of total protein were extracted from 6 g (902.6 mg), 22 g (2246.5 mg) and 40 g (3948.4 mg) of Heparin paste, respectively. If 902.6 mg of protein is extracted from 6 g of Heparin paste, then 3309.5 mg and 6017.3 mg of protein should be extracted from 22 g and 40 g of Heparin paste, respectively. Only 68% of the potential amount of protein is extracted when 22 g of Heparin paste is solubilised by Tris extraction buffer. Similarly, when 40 g of Heparin paste is solubilised, 66% of the potential amount of protein is extracted. Increasing levels of total clottable fibrinogen were extracted from 6 g (563.1 mg), 22 g (1303.7 mg) and 40 g (2786.3 mg) of Heparin paste, respectively. Again, if 6g of Heparin paste produces a yield of 563.1 mg of clottable fibrinogen, then 2064.7 mg and 3754.0 mg of clottable fibrinogen should be extracted from 22 g and 40 g of Heparin paste, respectively. Only 63% of the potential amount of clottable fibrinogen is extracted when 22 g of Heparin paste is solubilised by Tris extraction buffer. Similarly, when 40 g of Heparin paste is solubilised, 74% of the potential amount of clottable fibrinogen is extracted. This suggests that although all concentrations of Heparin paste can be solubilised to some extent using a constant volume of Tris buffer, protein and clottable fibrinogen extraction are less efficient with increasing amounts of Heparin paste. This indicates that the optimum amount of Heparin paste required for efficient extraction of protein and clottable fibrinogen is 6 g in 50 ml of Tris extraction buffer.

CONCLUSION

The removal of Tris, EDTA and ε-aminocaproic acid from the Tris buffer did not affect Heparin paste solubilisation and protein extraction compared to the control (Tris buffer). However, removal of EDTA from Tris buffer demonstrated increased levels of clottable fibrinogen compared to the control and other combinations of Tris buffer. Increased levels of clottable fibrinogen was also observed when EDTA was removed from the Citrate

buffer. This may be due to assay inhibition caused by inclusion of EDTA in these buffers. This also suggests that EDTA is not necessary for the extraction of fibrinogen from the Heparin paste.

The removal of ε-aminocaproic acid from both buffers appeared to reduce clottable fibrinogen and may therefore be an important component of the Heparin paste extraction buffers.

The inclusion of a salt such as NaCl was vital for the extraction of significant levels of fibrinogen and is therefore an essential component of the extraction solution. Tris buffer requires at least 0.2 M NaCl for maximum protein and clottable fibrinogen recovery. Like Tris buffer, it was demonstrated that the inclusion of NaCl in Citrate buffer and WFI was necessary for fibrinogen extraction. Citrate buffer requires at least 0.2 M NaCl for maximum protein and clottable fibrinogen recovery.

Variation of pH of the Tris buffer, demonstrated that all pH levels tested (6.0-9.0) were able to extract similar levels of fibrinogen compared to the control (pH 7.3). Variation of pH of the Citrate buffer demonstrated that pH 5.0 decreased extracted fibrinogen levels.

The amount of protein extracted by Tris buffer and Citrate buffer was comparable. However, Citrate buffer pH 7.3 was able to extract greater levels of clottable fibrinogen than Tris buffer. Since all other buffer components were the same, the presence of Na-Citrate (in place of Tris) in the Citrate buffer was able to stabilise fibrinogen to a greater extent than Tris. The optimal Na-Citrate concentration for the Citrate buffer is 20 mM. Na-Citrate at this concentration does not give the highest protein recovery but gives the greatest recovery of clottable fibrinogen. The average recovery of clottable fibrinogen is approximately 55% and approximately 100% for the Tris and Citrate buffers respectively. This emphasises the potential of the Citrate buffer for future extraction of fibrinogen from Heparin paste.

The disclosure of all references referred to herein are included herein by cross reference

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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CLAIMS:-

1. A method of obtaining a fibrinogen enriched preparation, the method including the following steps:-

(i) adding an effective amount of a sulphated polysaccharide (SPS) to a fibrinogen containing solution with to form a fibrinogen containing precipitate; and

(ii) extracting fibrinogen from the fibrinogen containing precipitate from step (i) with a solution containing at least 0.1 M, and preferably at least 0.2M, salt to obtain a fibrinogen enriched preparation.

2. A method as claimed in claim 1 in which the fibrinogen containing solution is a blood plasma fraction, preferably cryoprecipitate.

3. A method as claimed in claim 1 or claim 2 in which the solution includes at least one salt selected from the group consisting of chloride, phosphate and acetate salts.

4. A method as claimed in claim 3 in which the solution includes NaCl.

5. A method as claimed in claim 4 in which the NaCl is present at concentration of from about 0.1M to about 2.0M, preferably from about 0.2M to about 0.8M.

6. A method as claimed in any one of claims 1 to 5 in which the solution includes ε-aminocaproic acid.

7. A method as claimed in any one of claims 1 to 6 in which the SPS is a heparinoid selected from the group consisting of mucopolysaccharide polysulphate, pentosan polysulphate, chondroitin sulphate, dextran sulphate and heparin.

8. A method as claimed in any one of claims 1 to 7 in which the SPS is heparin.

9. A method as claimed in any one of claims 1 to 8 in which the SPS is added to the fibrinogen containing solution to provide a concentration of SPS of at least 0.15 mg/ml.

10. A method as claimed in any one of claims 1 to 9 in which the method further includes the step of treating the fibrinogen enriched preparation to remove SPS and/or plasminogen.

11. A method as claimed in any one of claims 1 to 10 in which the method further includes the step of subjecting the fibrinogen enriched preparation to a viral inactivation step.

12. A method as claimed in claim 11 in which the viral inactivation step involves heating and/or solvent detergent treatment.

13. A method as claimed in any one of claims 1 to 12 in which the fibrinogen is further purified from the fibrinogen enriched preparation by ion exchange chromatography, affinity chromatography, hydrophobic or gel permeation chromatography or a combination thereof.

14. A method of obtaining a preparation enriched for fibronectin or Factor XIII, the method comprising extracting fibronectin or Factor XIII from the fibrinogen enriched preparation obtained according to the method of claim 2.

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Nixon & Vanderhye P.C. (10/99)
(Domestic Non-Assigned/Foreign) **Page 1 of 2**

RULE 63 (37 C.F.R. 1.63)
INVENTORS DECLARATION FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PURIFICATION OF FIBRINOGEN

the specification of which (**check** applicable **box(s)**):

☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. Unassigned (Atty Dkt. No. 47-139)
☒ was filed as PCT international application No. PCT/AU99/00050 on 25 January 1999
and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance wit 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on whic priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	**Country**	**Day/Month/Year Filed**
PP1481	Australia	23 January 1998
PP1829	Australia	13 February 1998

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	**Date/Month/Year Filed**

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as th subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s): Application Serial No.	**Day/Month/Year Filed**	**Status: patented pending, abandoned**
PCT/AU99/00050	25 January 1999	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31362; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106 Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

1-8-00
1. Inventor's Signature: _____ Date: 10/1/2000
Inventor: Jerry (first) MI Kanellos (last) Australian (citizenship)
Residence: (city) Victoria (state/country) Australia
Post Office Address: 27 Myrtle Street, Clifton Hill, Victoria, Australia AUX
(Zip Code) 3068

2-8-00
2. Inventor's Signature: Teresa Martinelli Date: 11/7/2000
Inventor: Teresa (first) MI Martinelli (last) Australian (citizenship)
Residence: (city) Victoria (state/country) Australia
Post Office Address: 77 Ballantyne Street, Thornbury., Victoria, Australia AUX
(Zip Code) 3071

FOR ADDITIONAL INVENTORS, check box ☒ and attach sheet with same information and signature and date for each.

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the specification of which (**check** applicable **box(s)**):

☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. _____ (Atty Dkt. No. 47-139)
☒ was filed as PCT International application No. PCT/AU99/00050 on 25 January 1999
and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

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Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed	Status: patented pending, abandoned
PCT/AU99/00050	25 January 1999	

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3-00 3. Inventor's Signature: _____ Date: 11/7/00
Inventor: Grace (first) MI Demaria (last) Australian (citizenship)
Residence: (city) Victoria (state/country) Australia
Post Office Address: 5 Sydney Road, Brunswick, Victoria, Australia
(Zip Code) 3056

4-00 4. Inventor's Signature: _____ Date: 11/7/00
Inventor: Neil (first) MI Goss (last) Australian (citizenship)
Residence: (city) Victoria (state/country) Australia
Post Office Address: 39 Glenard Drive, Eaglemont, Victoria, Australia
(Zip Code) 3079